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Chicken macrophages infected with *Salmonella* (*S.*) Enteritidis or *S.* Heidelberg produce differential responses in immune and metabolic signaling pathways

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ABSTRACT

Protein kinases act in coordination with phosphatases to control protein phosphorylation and regulate signaling pathways and cellular processes involved in nearly every functions of cell life. *Salmonella* are known to manipulate the host kinase network to gain entrance and survive inside host cells. The effect of *Salmonella* infection on the host kinase network has been studied in mammalian cells, but information is largely lacking in chicken immune cells. Our previous study indicated that chicken macrophage cells respond differentially to different *Salmonella* strains. In order to better understand the interaction between chicken macrophages and *Salmonella,* we used a peptide array-based kinome analysis to identify cellular process and signaling pathways that may play a critical role in the outcome of *Salmonella* infection. The kinome assay was performed on chicken HD11 macrophages collected at 1.5, 3, and 7 h post-infection (hpi) with either *S.* Heidelberg or *S.* Enteritidis. A large number of peptides show significantly changed phosphorylation ($p \leq 0.05$) during the infection: 390, 449, and 575 peptides for *S.* Enteritidis and 185, 470, and 442 for *S.* Heidelberg at 1.5, 3, and 7 hpi, respectively. Many pathways involved in immunity, signal transduction, cellular process, and metabolism were significantly altered, in some case differentially, during the infection by the two Salmonella strains. Particularly, effects on lysosome process, iNOS, CARD9, NLRP3, and MAPK pathway provide significant insight to the inter play between pathogens and chicken macrophage cells during the infection.

1. Introduction

Salmonella enterica are Gram-negative facultative intracellular bacteria responsible for diseases ranging from self-limiting gastroenteritis (non-typhoidal *Salmonella*) to life-threatening typhoid fever (serovar Typhi) in humans (Scallan et al., 2011). In chickens, infections with host specific serovars *S. Gallinarum* and *S. Pullorum* are deadly, causing septicemia fowl typhoid and pullorum disease; while infections with non-host specific serovars such as *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg display little clinical symptoms (Barrow and Freitas Neto, 2011). Poultry products contaminated with these non-host specific *Salmonella* serovars, however, are the important source of zoonotic pathogens for human foodborne illness (Scallan et al., 2011). Reduction of colonization of *Salmonella* in chickens, thereby decreasing the incidence of *Salmonella* contamination in meat and eggs, is a critical undertaking to produce poultry products which are safe for human consumption.

Different hosts respond differently to *Salmonella* infection. Although chicken immune cells produce significant inflammatory immune response when exposed to *Salmonella*, including increasing production of inflammatory cytokines, nitric oxide, and reactive oxygen species, by innate immune cells (Kaiser et al., 2000; He et al., 2012; Setta et al., 2012); unlike human, chickens infected with most non-host specific *Salmonella* serovars can live normally. The mechanism of tolerance which allows chickens to harbor *Salmonella* commensally in intestine and sometimes even in internal organs, such as liver and spleen, remains unclear. However, *Salmonella* are well known to have evolved highly complex strategies to circumvent host immune defense mechanisms (Haraga et al., 2008). The most widely studied is the type III secretion system (T3SS) which produces nearly 40 different virulence effectors to enable *Salmonella* invasion, survival, and replication inside the host cells (Haraga et al., 2008; Ibarra and Steele-Mortimer, 2009; Malik-Kale et al., 2011).

Protein kinases, along with phosphatases, control protein

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phosphorylation and regulate signaling pathways and cellular processes involved in nearly every aspect of cell life, including metabolism, transcription, cell-cycle, apoptosis, cell movement, protein interaction and localization, enzyme activity, and immune function (Johnson, 2009). *Salmonella* infection of host cells (RAW264.7 and HeLa cells) have been shown to causes extensive changes in protein phosphorylation (Rogers et al., 2011; Imami et al., 2013). Although majority of the changes are induced by cellular defense mechanisms to control and eliminate infection, evidence also shows that intracellular bacteria, such as *Salmonella*, manipulate the host kinase network to benefit their intracellular survival (Rogers et al., 2011).

Macrophages are important innate immune cells that play a central role in the first line defense against microbial infection, in which they detect, phagocytize, and produce microbicidal substances, including reactive radical oxygen species (ROS), nitric oxide, lysozyme, and proteolytic enzymes, to kill the infectious agents. Although chicken macrophages are equipped with effective antimicrobial mechanisms that can be readily deployed in response to microbial stimulations (Babu et al., 2006; Okamura et al., 2005; Withanage et al., 2005; He et al., 2006, 2011), many *Salmonella* strains, especially *S*. Enteritidis, are able to survive inside the macrophages (He et al., 2012, 2013). The interaction between chicken macrophages and *Salmonella* as well as intracellular survival of *Salmonella* in chicken macrophages remains poorly understood. Recently, a species-specific peptide array-based kinome technique has been developed for agricultural species, such as bovine, turkey, and chicken (Jalal et al., 2009; Arsenault et al., 2012, 2013a, 2013b, 2014), to study signaling pathway involved in host-microbe interaction and host immune response. Using chicken-specific kinome array assay, *Salmonella* have been found to extensively modulate host immune signaling pathways, leading to establish a persistent colonization of chicken gastrointestinal tract (Kogut and Arsenault, 2015; Kogut et al., 2016). These studies reveal a complex effect of *Salmonella* infection on host kinase network at the tissue level, which reflects the outcome of different types of cells presented at the experimental samples. Cell line on the other hand provides pure population of a specific cell type that allows us to study the interaction of *Salmonella* with a particular cell population. As macrophages present at the gut tissue, spleen, liver, and blood circulation, they phagocytise *Salmonella* and thus play a critical role in control of infection. However, due to *Salmonella* being able to survive inside macrophage cells, macrophage cells have also shown to be responsible for systemic infection in chickens (Chappell et al., 2009). Using the chicken macrophage cell line HD11, the kimone array analysis can be a powerful tool to undercover critical signaling pathways involved in *Salmonella* interacting with macrophages and intracellular survival, which will provide new information needed for managing *Salmonella* in poultry. Here, for the first time, we used this technique to identify important pathways affected by different *Salmonella* strains *S*. Enteritidis and *S*. Heidelberg infections in chicken macrophage cell line HD11.

2. Experimental procedures

2.1. Bacteria

Salmonella Enteritidis and *S.* Heidelberg used in the present study were initially field isolates from poultry farms and were serotyped by the National Veterinary Services Laboratory (Ames, IA, USA). These isolates were selected to resist Carbonicillin-novobiocin (C-N) and have been used in our previous studies (He et al., 2012). *Salmonella* stocks were stored in 75% trypticase soy broth (TSB) + 25% sterile glycerol in aliquots of 1×10^9 colony forming units (cfu) at −80 °C until used. The aliquots of the stocks were cultured overnight at 41 °C in BD's Tryptic Soy Broth (TSB), aliquots of the overnight cultures were transferred to a fresh TSB and cultured at 41 °C for 4 h to reach exponential growth phase, and the bacteria were pelleted using a centrifuge, washed, and resuspended in PBS at a final concentration of $\sim 1 \times 10^9$ (cfu, colony-forming unit)/ml. The viable cell concentrations of *S*. Enteritidis and *S*. Heidelberg were determined by colony counts on BD's Difco's xylose-lysine tergitol 4 (XLT4) agar plates containing C-N.

2.2. Chicken macrophage HD11 cells

The MC29 virus-transformed chicken macrophage cell line HD11 (Beug et al., 1979) was maintained in complete Dulbecco's Modified Eagles Medium (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 µg streptomycin/ml), and 1.5 mM L-glutamine at 39 °C, 5% CO_2, and 95% humidity. Aliquots of cell suspension (2×10^6 cells/ml) was seeded into each well at 1 ml/well in 12-well plates (BD) and allowed to grow to about 85% confluence (~36 h) before used for infection.

2.3. Infection of HD11 cells with Salmonella

Culture medium was removed from the HD11 cells and infected with 500 µl of *Salmonella* suspensions ($\sim 5 \times 10^8$ cfu/ml in plain DMEM) were added to each well with multiplicity of infection (MOI) at about 50:1 and three replicate wells for each serovar and incubated for 1 h at 39 °C in a 5% CO2 humidified incubator. At 1 hpi, the infection medium was removed and the cells were washed once with plain DMEM, treated with 100 µg/ml of gentamicin sulfate for 30 min and followed by 25 µg/ml of gentamicin sulfate in complete DMEM to kill extracellular bacteria. At 30 min, 2, and 6 h after gentamicin treatment, infected cells (designated as 1.5, 3, and 7 hpi, respectively) were washed once with cold PBS buffer and collected and pelleted in a centrifuge at 300 × g. Cell pellets were snap frozen in liquid nitrogen, and then stored at a −80 °C freezer. Live intracellular *Salmonella* at each hpi were confirmed in replicate wells by lysing HD11 s and plating on XLT4 agar with C-N.

Intracellular viable *Salmonella* were determined at 1.5 hpi as described previously (He et al., 2013) to make sure the infection. Briefly, infected cells were washed twice with PBS and lysed for 10 min in 1% Triton X-100 (in PBS). Serial 1:10 dilutions of the lysates were plated onto XLT4 agar plates containing C and N and incubated at 41 °C for 24 h. Colonies were counted to determine the cfu of intracellular viable bacteria. For the present study, the cfu of intracellular viable *Salmonella* at 1.5 hpi from the identical replicate plates were averaging 2.5×10^6 and 2.1×10^6 for SE and SH, respectively.

2.4. Nitric oxide production assay

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Greiss assay (Green et al., 1982). HD11 cells were infected with either *S.* Enteritidis or *S.* Heidelberg as described above. At 1.5, 3, and 7 hpi, aliquots of 100 µl culture supernatant from each well were transferred to the wells of a new flat-bottom 96-well plate and mixed with 50 µl of 1% sulfanilamide and 50 µl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution) sequentially. After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD_{595}) of each well using a microplate reader. Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in the cell-free medium.

2.5. Peptide array experimental protocol

Cells were removed from the −80C freezer and immediated lysed in 100 uL of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl,1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4,1 mM NaF,1 ug/ml leupeptin, 1 g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride [**PMSF**]) (all products from Sigma-Aldrich, St Louis, MO, unless indicated).

Lysate was incubated on ice for 10 min then spun in a

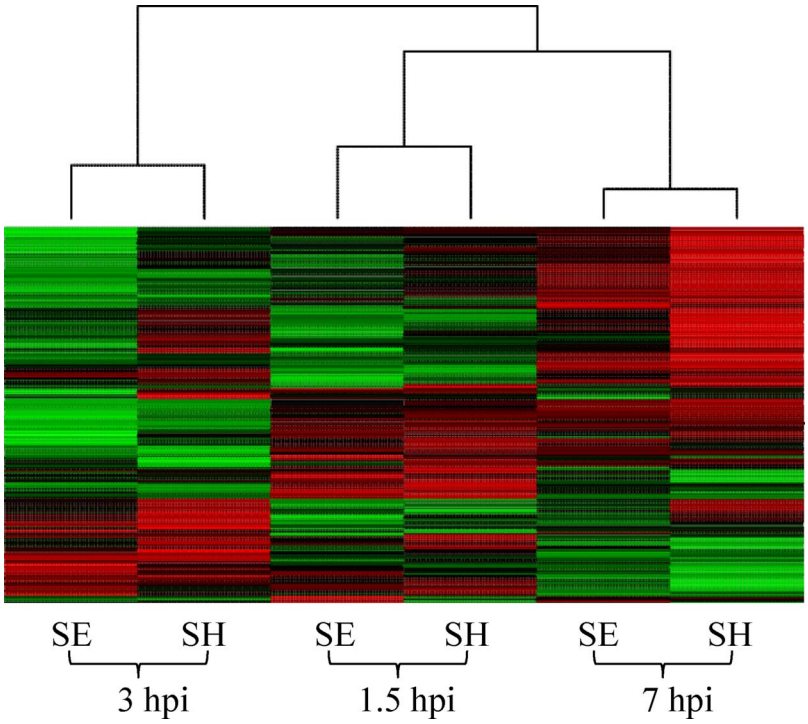

Fig. 1. Hierarchical clustering analysis of peptide phosphorylation changes in HD11 macrophage-like cells after infection with two *Salmonella* strains, *S.* Enteritidis and *S.* Heidelberg, at MOI 50. HD11 cell lysates were collected after 1.5, 3, and 7 h post infection (hpi). The host kinase network was analyzed using a chicken-specific kinome peptide array. Response of the infected samples was subtracted from the response of uninfected controls (3 replicates). The data represent phosphorylation changes of 771 peptides.

microcentrifuge at 14,000 g for 10 min at 4 °C. The final protein concentration was measured (Pierce Modified Lowry Protein Assay Kit) and adjusted to 1.5 mg/ml. A 70 ul aliquot of the resultant supernatant was mixed with 10 ul of activation mix (50% glycerol, 500 uM ATP [New England BioLabs, Ipswich, MA], 60 mM MgCl2, 0.05% [vol/vol] Brij 35, 0.25 mg/ml bovine serum albumin [BSA]) in a new microcentrifuge tube. Peptide array production was done on a contract basis with JPT Peptide Technologies (Berlin, Germany). 771 unique kinase substrate target peptide sequences were printed in replicate 9 times.

A 25 × 60 mm, 85 uL glass lifter slip was applied to the microarray to sandwich and disperse the applied lysate. Eighty µL of the mixture was applied to the peptide microarray, ensuring that no bubbles were present in the pipette tip or array slide. Slides were incubated for 2 h in a humidity chamber: a sealed container containing a small amount of water (not in contact with the arrays) within an incubator at 37 °C at 5% CO_2. Arrays were removed from the incubator and humidity chamber and placed in a 50 ml centrifuge tube containing phosphate-buffered saline (**PBS**)–1% Triton. The arrays were submerged repeatedly until the lifter slip slid off the array. Arrays were then submerged in 2 M NaCl-1% Triton and agitated for a minimum of 10 s. This process was then repeated with fresh 2 M NaCl-1% Triton. Arrays were submerged in ddH20 and agitated for a minimum of 10 s. Array slides were removed from the ddH_2O and submerged in phospho-specific fluorescent ProQ Diamond Phosphoprotein Stain (Life Technologies, Carlsbad, CA) in a dish and placed on a shaker table at 50 rpm for 1 h. The dish was covered to protect the fluorescent stain from light. Arrays were then placed in a new dish and submerged in destaining solution containing 20% acetonitrile (EMD Millipore Chemicals, Billerica, MA) and 50 mM sodium acetate (Sigma) at pH 4.0 for 10 min with agitation at 50 rpm. The petri dish was covered to protect the stain from light. This process was repeated 2 times. A final wash was done with distilled deionized H_2O.

Arrays were placed in 50 ml centrifuge tubes with a crumpled kim wipe in the bottom. The tubes containing the arrays were then centrifuged at 300 ×g for 2 min to remove any moisture from the array. Arrays were scanned using a Tecan PowerScanner microarray scanner (Tecan Systems, San Jose, CA) at 532–560 nm with a 580-nm filter to detect dye fluorescence.

2.6. Statistical and data analysis

Images were generated and the spot intensity signal was collected as the mean of pixel intensity using local feature background intensity calculation with the default scanner saturation level.

Images were gridded using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale CA), and the spot intensity signal was collected as the mean of pixel intensity using local feature background intensity calculation with the default scanner saturation level. The resultant data was then analyzed by the PIIKA2 peptide array analysis software (Trost et al., 2013, p. 2). Briefly, the resulting data points were normalized to eliminate variance due to technical variation, for example, random variation in staining intensity between arrays or between array blocks within an array. Variance stabilization normalization was performed. Note: as the arrays were printed with triplicate peptide blocks there are 3 values for each peptide. Using the normalized data set comparisons between treatment and control groups were performed, calculating fold change and a significance *p*-value. The *p*-value is calculated by conducting a one-sided paired *t*-test between treatment and negative control values for a given peptide. The resultant fold change and significance values were then used to generate optional analysis (heatmaps, hierarchical clustering, principal component analysis, pathway analysis).

3. Results

3.1. Cluster analysis

The kinome data were analyzed by Hierarchical clustering analysis to identify global peptide phosphorylation change patterns associated with bacterial stains and durations of infection (hpi). The result, as shown in the heatmap (Fig. 1), clearly demonstrated a distinctive segregation associated with the duration of infection (hpi), despite significant differences existing between the two *Salmonella* strains. During the progress of infection, *Salmonella* invoked extensive and dynamic events of protein phosphorylation/dephosphorylation activity associated with the cellular kinase network; these changes were more closely associated with stage of infection than with the strains of

Table 1
Effect of *Salmonella* infection on peptide phosphorylation*.

Phosphorylation	1.5 hpi		3 hpi		7 hpi	
	SE	SH	SE	SH	SE	SH
↑	206	185	178	257	327	249
↓	186	190	271	213	248	194
Total	392	375	449	470	575	443

SE	SH	1.5 hpi	3 hpi	7 hpi
↑	↑	87	126	226
↓	↓	108	141	159
↑		119	52	101
↓		78	130	89
	↑	98	131	23
	↓	82	72	35
↑	↓	4	4	2
↓	↑	26	14	1

Arrow indicates increase (↑) or decrease (↓) in phosphorylation. SE: *S.* Enteritidis; SH: *S.* Heidelberg.

* Numbers are the peptides with significant ($p \leq 0.05$) phosphorylation change.

Salmonella. However, the data also indicated unique pathogen specific interaction with the host, which was evidenced by the difference in phosphorylation change pattern between the two *Salmonella* serovars within each time point (hpi).

3.2. Peptide phosphorylation: similarity and difference between cells infected by S. Enteritidis and S. Heidelberg

The data were separated into groups (Table 1) based on hpi, *Salmonella* strains, and increase or decrease in phosphorylation to provide an easier view of the dynamic changes in protein phosphorylation in HD11 cells during *Salmonella* infection (see details in the Additional File 1). The peptide array contains a total of 771 peptides that were derived from phosphorylation sites of 572 proteins, with some proteins having multiple peptides to cover different phosphorylation sites. At 1.5 hpi, almost half of the total peptides showed significant changes in phosphorylation status; as the infection progressing, the numbers of peptides with significant phosphorylation changes continued to increase for both *Salmonella* strains. However, the effects on peptide phosphorylation between *S.* Enteritidis and *S.* Heidelberg were more divergent at early stage of infection from 1.5 to 3 hpi. At 1.5 hpi, 197 out of 392 (197/392) peptides showing significant phosphorylation change were uniquely associated with *S.* Enteritidis and 180 out of 375 (180/375) with *S.* Heidelberg. At 3 hpi, 182/449 and 203/470 were specifically associated with *S.* Enteritidis and *S.* Heidelberg, respectively. The percentage of these *Salmonella* strain specific peptides with significant phosphorylation change decreased significantly at 7 hpi, with 190/575 for *S.* Enteritidis and 58/443 for *S.* Heidelberg. The diminishing divergence between the two strains of *Salmonella* as infection progressed was also evidenced by the increased number of peptides sharing the same pattern of phosphorylation change; these peptides accounted for 195/392, 267/449, and 385/575 for *S.* Enteritidis and 195/375, 267/470, and 385/443 for and *S.* Heidelberg, at 1.5, 3, and 7 hpi, respectively. These data suggested that there was a significant strain-specific host cell response and this strain-specific host response largely occurred during the initial phase of infection and was gradually converged to more common response.

3.3. Pathways significantly affected by Salmonella infection

The changes of peptide phosphorylation catalyzed by the cell lysates harvested at each time points during the infection reflect the work of cellular kinome network. The peptides showing significant changes of phosphorylation were subjected to the KEGG pathway analysis using the STRING database (Kanehisa et al., 2012) to predict pathways significantly affected by infection. These pathways and their participating member proteins showing significant phosphorylation change are most likely involved in the host-pathogen interaction. KEGG pathways with significant change ($p \leq 0.05$) are listed in Table 2. Upon infection, both *Salmonella* strains incited significant changes in an extensive number of pathways. These pathways are involved in vast range of cellular functions, including both innate and adaptive immune systems, cellular defense mechanism, signaling cascades critical for gene expression and regulation, pathways responsible for cell surface and intracellular structures, and metabolic processes.

3.4. Differential induction of nitric oxide by S. Enteritidis and S. Heidelberg

Nitric oxide (NO) production from HD11 cells infected with *S.* Enteritidis and *S.* Heidelberg were measured at 1.5, 3, and 7 hpi (Fig. 2A). No measurable amount of NO was produced at 1.5 hpi in cells infected with either *Salmonella* strains. At 3 hpi, production of NO was numerically higher in cells infected with *S.* Heidelberg. However, at 7 hpi, production of NO was significantly higher in cells infected with *S.* Heidelberg (18.2 ± 6.0 μM) than cells infected with *S.* Enteritidis (6.4 ± 3.8 μM), indicating a differential effect of the two *Salmonella* strains on HD11 cell NO response.

4. Discussion

4.1. Differential effect of S. Enteritidis and S. Heidelberg on lysosome and phagosome processes

A notable difference between the two *Salmonella* strains is that at each of three time points during the infection, *S.* Enteritidis, but not *S.* Heidelberg, infected cells produced significant changes in lysosome pathway (Table 2). Similarly, the phagosome process of HD11 cells was also less affected by *S.* Heidelberg infection than by *S.* Enteritidis (Table 2). Significantly affected peptides of Lysosome and phagosome processes are listed in Table 3. Lysosomes contain a large number of catabolic enzymes and they play a key role in degrading pathogens when fused with the pathogen-containing phagosomes or autophagosomes in macrophage cells. However, *Salmonella* such as Typhimurium can disrupt the formation of phagolysosome to avoid exposure to harmful lysosomal contents (Brumell and Grinstein, 2004). It is not clear whether *Salmonella* act in the similar fashion to interfere the phagolysosome maturation process in chicken macrophages. However, the results suggest that *S.* Enteritidis is more effective in manipulating the host lysosome activation process than *S.* Heidelberg, which in turn may contribute the higher survival rate of *S.* Enteritidis in chicken macrophage HD11 cells as reported previously (He et al., 2012).

4.2. Salmonella infection induces iNOS activity by down-regulating iNOS phosphorylation

Inducible nitric oxide synthase (iNOS) is the enzyme that catalyzes the formation of nitric oxide (NO) in macrophages from L-arginine, oxygen and NADPH in response to stimulation by microbial products (MacMicking et al., 1997). Studies have indicated that NO plays important role in controlling the intracellular bacterial pathogens such as *Salmonella* Typhimurium (Mastroeni et al., 2000; Alam et al., 2002), even though its effectiveness is debatable due to the factor that *Salmonella* secretes T3SS effector proteins which can suppress iNOS activity (Das et al., 2009) or insulate themselves from exposure to reactive nitrogen species (RNS) (Chakravortty et al., 2002). Additionally, *Salmonella* can also produce enzymes (lavohemoglobin Hmp, flavorubredoxin NorV, and cytochrome *c* nitrite reductase NrfA) to neutralize NO (Bang et al., 2006; Mills et al., 2008). In the present study, *Salmonella* infection was found to significantly reduce the phosphorylation of chicken macrophage iNOS (Fig. 2B). The peptide of chicken iNOS used

Table 2
KEGG pathways that were significantly affected by *Salmonella* infection.

GO_id	Pathway Name	1.5 hpi				3 hpi				7 hpi			
		SE		SH		SE	SH	SE	SH	SE		SH	
		#	*p*-value	#	*p*-value	#	*p*-value	#	*p*-value	#	*p*-value	#	*p*-value
Immune system													
hsa04612	Antigen processing and presentation	5	6.6E-03	5	5.0E-03	7	2.2E-04	7	2.8E-04	8	9.2E-05	4	4.3E-02
hsa04062	Chemokine signaling pathway	29	2.2E-21	26	1.3E-18	34	5.9E-26	33	2.9E-24	38	3.7E-28	32	4.6E-24
hsa04623	Cytosolic DNA-sensing pathway	4	2.0E-02	3	9.1E-02	5	4.9E-03	3	1.3E-01	5	9.7E-03	6	6.0E-04
hsa04664	Fc epsilon RI signaling pathway	21	5.9E-21	19	1.5E-18	20	1.0E-18	25	1.9E-25	24	8.4E-23	18	2.3E-16
hsa04666	Fc gamma R-mediated phagocytosis	19	1.1E-16	20	2.6E-18	19	7.4E-16	24	5.7E-22	26	2.6E-23	20	2.2E-17
hsa04670	Leukocyte transendothelial migration	19	5.7E-15	12	8.4E-08	18	4.7E-13	21	3.8E-16	20	5.7E-14	15	4.2E-10
hsa04650	Natural killer cell mediated cytotoxicity	17	1.0E-11	21	1.6E-16	18	5.2E-12	24	2.8E-18	26	3.2E-19	23	9.8E-18
hsa04621	NOD-like receptor signaling pathway	8	3.3E-06	9	1.5E-07	11	2.8E-09	12	2.6E-10	11	1.7E-08	10	3.2E-08
hsa04622	RIG-I-like receptor signaling pathway	4	3.3E-02	4	2.8E-02	7	2.2E-04	10	4.2E-07	9	1.2E-05	6	1.4E-03
hsa04620	Toll-like receptor signaling pathway	17	1.6E-13	20	1.6E-17	22	1.0E-18	29	1.6E-27	31	9.7E-29	25	5.2E-23
Signal transduction													
hsa04920	Adipocytokine signaling pathway	23	2.6E-25	18	2.7E-18	17	8.2E-16	21	4.6E-21	20	1.3E-18	19	8.3E-19
hsa04020	Calcium signaling pathway	15	6.2E-08	16	3.8E-09	20	9.5E-12	18	1.2E-09	19	1.5E-09	17	3.1E-09
hsa04060	Cytokine-cytokine receptor interaction	11	2.5E-03	13	1.0E-04	10	1.5E-02	11	7.0E-03	17	1.7E-05	15	2.4E-05
hsa04012	ErbB signaling pathway	30	2.8E-33	30	5.8E-34	32	3.2E-35	35	1.9E-39	37	8.2E-41	32	2.1E-35
hsa04340	Hedgehog signaling pathway	4	1.7E-02	4	1.4E-02	4	2.3E-02	5	4.5E-03	5	7.8E-03	5	3.4E-03
hsa04910	Insulin signaling pathway	38	4.8E-38	32	1.3E-30	37	3.2E-35	44	1.6E-44	44	2.3E-42	40	8.4E-40
hsa04630	Jak-STAT signaling pathway	15	1.3E-08	11	1.6E-05	18	9.6E-11	21	2.3E-13	25	3.2E-16	17	5.4E-10
hsa04010	MAPK signaling pathway	43	4.4E-32	44	5.4E-34	43	3.1E-30	50	1.6E-37	57	1.0E-42	41	2.0E-28
hsa04150	mTOR signaling pathway	18	3.7E-20	16	2.0E-17	18	2.5E-19	20	3.6E-22	20	3.5E-21	19	3.3E-21
hsa04722	Neurotrophin signaling pathway	34	2.8E-33	35	3.0E-35	37	4.9E-36	38	3.6E-37	41	2.6E-39	35	9.6E-34
hsa04070	Phosphatidylinositol signaling system	8	3.0E-05	7	2.6E-05	8	5.6E-05	9	9.4E-06	10	3.4E-06	7	3.6E-04
hsa03320	PPAR signaling pathway	6	1.1E-03	6	8.2E-04	9	3.4E-06	9	4.8E-06	7	6.8E-04	6	1.4E-03
hsa04350	TGF-beta signaling pathway	6	2.5E-03	6	1.8E-03	9	1.2E-05	9	1.7E-05	9	4.8E-05	7	5.6E-04
hsa04370	VEGF signaling pathway	19	2.5E-18	19	1.3E-18	17	6.0E-15	21	5.5E-20	24	6.1E-23	18	1.9E-16
hsa04310	Wnt signaling pathway	19	9.2E-13	18	4.2E-12	19	5.5E-12	22	9.4E-15	23	1.5E-14	17	3.0E-10
Cellular process													
hsa04210	Apoptosis	14	4.7E-11	12	4.1E-09	13	2.1E-09	18	6.6E-15	19	4.4E-15	16	6.3E-13
hsa04520	Adherens junction	11	1.0E-08	17	1.50E-16	12	2.1E-09	14	1.5E-11	17	2.5E-14	12	1.6E-09
hsa04110	Cell cycle	10	2.0E-05	10	1.2E-05	10	4.3E-05	9	3.2E-04	10	1.7E-04	8	1.1E-03
hsa04144	Endocytosis	21	1.4E-12	19	4.3E-11	23	1.6E-13	26	4.3E-16	25	7.4E-14	18	2.6E-09
hsa04510	Focal adhesion	38	3.3E-31	32	1.4E-24	40	6.1E-32	42	7.9E-34	47	1.2E-37	34	3.2E-25
hsa04540	Gap junction	9	8.5E-06	11	5.1E-08	12	2.2E-08	12	3.0E-08	14	1.6E-09	11	1.8E-07
hsa04142	Lysosome	6	1.5E-02		N/S	7	5.7E-03		N/S	8	3.6E-03		N/S
hsa04146	Peroxisome	7	2.4E-04	11	1.6E-08	8	5.6E-05	9	9.4E-06	7	1.1E-03	8	4.8E-05
hsa04145	Phagosome	7	8.8E-03		N/S	8	3.8E-03	9	1.1E-03	9	2.9E-03		N/S
hsa04141	Protein processing in ER	14	1.70E-07	12	4.1E-06	14	5.3E-07	16	2.1E-08	14	4.1E-06	14	3.9E-07
hsa04810	Regulation of actin cytoskeleton	27	5.5E-18	25	1.9E-16	33	4.3E-23	31	1.7E-20	35	5.6E-23	24	3.1E-14
hsa04530	Tight junction	12	6.8E-07	11	2.8E-06	13	2.6E-07	12	2.7E-06	14	2.7E-07	11	9.3E-06
Bacterial Infection													
hsa05100	Bacterial invasion of epithelial cells	14	1.6E-12	13	1.6E-11	15	4.1E-13	15	6.0E-13	19	3.8E-17	13	7.8E-11
hsa05130	Pathogenic Escherichia coli infection	8	1.9E-06	8	1.2E-06	10	2.0E-08	9	4.0E-07	10	9.8E-08	6	3.2E-04
Metabolism													
hsa00020	Citrate cycle (TCA cycle)	3	1.5E-02	3	1.3E-02	4	2.0E-03	5	1.9E-04	5	3.4E-04	4	1.8E-03
hsa00061	Fatty acid biosynthesis	2	7.2E-03	2	6.1E-03	2	8.0E-03	2	8.5E-03	2	1.1E-02	7	9.3E-06
hsa00071	Fatty acid metabolism	6	7.8E-05	7	4.1E-06	7	1.1E-05	7	1.4E-05	2	1.9E-02		N/S
hsa00051	Fructose and mannose metabolism	7	1.6E-06	4	2.7E-03	8	1.6E-07	9	1.0E-08	8	5.4E-07	6	3.4E-05
hsa00052	Galactose metabolism	6	4.1E-06	4	9.1E-04	9	4.3E-10	7	4.0E-07	8	4.8E-08	6	6.1E-06
hsa00010	Glycolysis/Gluconeogenesis	11	2.4E-09	11	1.3E-09	12	4.0E-10	15	1.1E-13	15	7.1E-13	11	4.5E-09
hsa00562	Inositol phosphate metabolism	6	3.7E-04	7	2.6E-05	7	6.7E-05	7	8.6E-05	8	2.3E-05	6	5.1E-04
hsa01100	Metabolic pathways	34	2.5E-05	35	3.0E-06	38	6.3E-06	42	4.3E-07	46	4.3E-07	37	8.3E-06
hsa00030	Pentose phosphate pathway		N/S		N/S	4	1.5E-03	5	1.3E-04	6	1.7E-05		N/S
hsa00640	Propanoate metabolism	4	2.3E-03	5	1.3E-04	5	2.5E-04	6	2.3E-05	7	3.4E-06	5	2.4E-04
hsa00620	Pyruvate metabolism	4	4.7E-03	6	2.6E-05	5	6.7E-04	5	7.8E-04	6	1.5E-04	5	6.0E-04
hsa00500	Starch and sucrose metabolism	5	1.5E-03	5	1.1E-03	6	2.5E-04	7	3.2E-05	9	6.7E-07	6	2.3E-04
hsa00280	Val, Leu and Ile degradation	3	4.9E-02	3	4.4E-02	4	1.0E-02	4	1.2E-02	5	2.8E-03		N/S

in the array contains the phosphorylation site Y148 which corresponds to Y151 of human iNOS. Phosphorylation at Y151 of human iNOS is known to inhibit the iNOS activity (Hausel et al., 2006). Our results demonstrate for the first time that, in response to microbial infection, chicken macrophage iNOS activity can be stimulated not only by increased iNOS gene expression (Elsheimer-Matulova et al., 2015), but also by protein dephosphorylation to remove the inhibition. The result strongly indicates under normal condition, iNOS may be phosphorylated to keep the activity in check; while, in response to *Salmonella* infection, the host cells can quickly boost the iNOS activity through modifying its protein phosphorylation state. This rapid activation of existing iNOS before increasing de novo expressed iNOS may be one of the host defense strategies to deploy NO against microbial infection.

However, the activation of iNOS by dephosphorylation alone cannot explain why *S*. Enteritidis induced significantly less NO production in

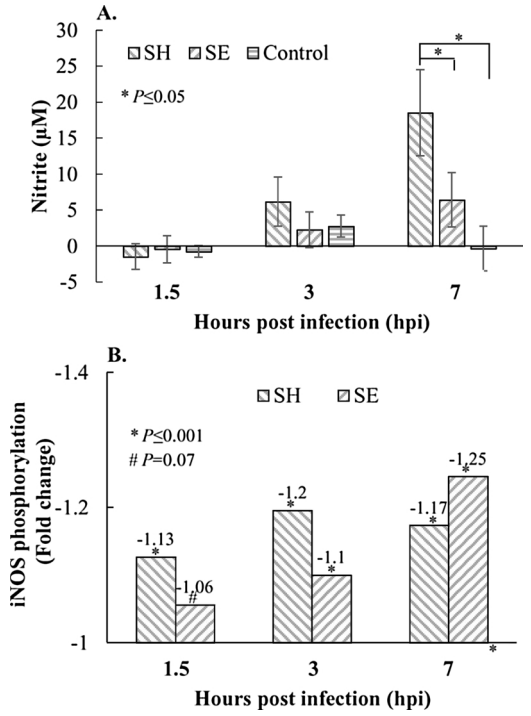

Fig. 2. Nitric oxide (NO) production and phosphorylation change of iNOS peptide in HD11 macrophage-like cells after infection with two *Salmonella* strains, S. Enteritidis and S. Heidelberg. A. NO production measured as nitrite in cell culture supernatant); B. Fold change of phosphorylation at Y148 residue of the iNOS peptide generated by peptide array from *Salmonella* infected HD11 cells harvested at 1.5, 3, and 7 hpi.

the infected HD11 cells than *S*. Heidelberg as observed in the present study (Fig. 2A), since both strains similarly down-regulated iNOS phosphorylation (Fig. 2B). The similar outcome of NO production between HD11 cells infected by the two *Salmonella* strains was also observed in our previous study (4) where *S*. Heidelberg infection stimulates significant amount of NO, whereas little NO was induced by *S*. Enteritidis. This discrepancy cannot be readily explained. Our hypothesis is that the outcome of NO production were determined by opposing activities from the host cells and the pathogens; in this case, *S*. Enteritidis may have more effective NO neutralizing capability than *S*. Heidelberg to obliterate the NO response of chicken macrophage cells. This ability to neutralize NO production may be one contributing factor that facilitates *S*. Enteritidis to survival inside chicken macrophage cells (He et al., 2012).

4.3. *Salmonella* infection activates multiple toll-like receptor (TLR) pathways

Phosphorylation status of a large number of peptides representing proteins in the TLR pathway were significantly altered by cells infected with both *Salmonella* strains (Table 4). This is not surprising because TLRs are the most important pattern recognition receptors in the innate immune system that recognize pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). Many chicken TLRs have been identified, including homologues to human TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, and chicken specific TLR15 and TLR21 (Keestra et al., 2013). Phosphorylation of TLR5 that recognizes *Salmonella*'s flagellin is significantly affected at 3 hpi for both *Salmonella* strains. TLR4 interacts with lipopolysaccharide (LPS) of Gram-negative bacteria. Unfortunately, TLR4 was not included in the peptide array due to lack of

Table 3
Proteins involved in endocytosis and cellular defense mechanisms whose phosphorylation were significantly (p-value ≤ 0.05) affected by *Salmonella* infection at 1.5, 3, and 7 hpi.

S. Enteritidis						S. Heidelberg					
1.5 hpi		3 hpi		7 hpi		1.5 hpi		3 hpi		7 hpi	
Endocytosis											
ARRB1	MET	AP2M1	KDR	AP2M1	KDR	AP2M1	NTRK1	AP2M1	KDR	AP2M1	PDGFRA
ARRB2	NTRK1	ARRB1	NTRK1	ARRB1	KIT	ARRB1	PDGFRA	ARRB2	MET	ARRB1	RAB4A
CAV1	PDGFRA	ARRB2	PDGFRA	CAV1	MET	ARRB2	RAB7A	CBL	NTRK1	CSF1R	RAB7A
CSF1R	RAB4A	CAV1	RAB5A	CBL	NTRK1	CAV1	RHOA	CSF1R	PDGFRA	EGFR	SH3KBP1
EEA1	RHOA	CBL	RHOA	CSF1R	PDGFRA	CSF1R	SMAD2	EEA1	RAB4A	FGFR2	SMAD2
EGFR	SH3KBP1	EEA1	SH3KBP1	EEA1	PIP5K1C	EGFR	SRC	EGFR	RAB5A	FGFR4	
FGFR2	SMAD2	EGFR	SMAD2	EGFR	RAB4A	FGFR2		FGFR2	RAB7A	GIT2	
FGFR3	SRC	FGFR2	STAM	FGFR2	RAB7A	FGFR3		FGFR3	RHOA	GRK5	
FGFR4		FGFR3	STAM2	FGFR3	RHOA	GRK5		FGFR4	SH3KBP1HSPA8		
GIT2		FGFR4	TRAF6	FGFR4	SH3KBP1	HSPA8		GIT2	SMAD2	KDR	
GRK5		GIT2		GIT2	SMAD2	KDR		GRK5	STAM	KIT	
HSPA8		GRK5		GRK5	SRC	KIT		HRAS	STAM2	MET	
KDR		HSPA8		HSPA8		MET		HSPA8	TRAF6	NTRK1	
Lysosome											
CTSB	IGF2R	CTSB	IGF2R	CTSB	GALC	CTSO		CTSB	M6PR	CTSB	M6PR
CTSO	M6PR	CTSL1	LAMP3	CTSL1	IGF2R	CTSS		CTSS		CTSO	
CTSS		CTSO	M6PR	CTSO	LAMP3	IGF2R		IGF2R		CTSS	
GALC		GALC		CTSS	M6PR			LAMP3		GALC	
Peroxisome											
ACAA1	HSD17B4	ACAA1	NOS2	ACAA1	PECR	ACAA1	CROT	ACAA1	HSD17B4	ACOX1	NOS2
ACOX1		ACSL4	PECR	ACOX1		ACOX1	HACL1	ACOX1	NOS2	ACSL4	PECR
ACSL5		ACSL5		ACSL5		ACSL4	HSD17B4	ACSL4	PECR	ACSL5	
ACSL6		ACSL6		CAT		ACSL5	NOS2	ACSL6		CAT	
CAT		CAT		HSD17B4		ACSL6	PECR	CAT		CROT	
HACL1		HSD17B4		NOS2		CAT		CROT		HSD17B4	
Phagosome											
CTSS	PIKFYVE	CTSL1	PIKFYVE	CTSL1	RAB7A	CTSS		CTSS	RAB5A	CTSS	TLR6
DC1L1	THBS1	DC1L1	RAB5A	CTSS	THBS1	DC1L1		DC1L1	RAB7A	M6PR	
EEA1		EEA1	THBS1	DC1L1	THBS3	RAB7A		EEA1	THBS1	RAB7A	
M6PR		M6PR		EEA1	TLR6	THBS1		M6PR	THBS3	THBS1	
NCF2		NCF2		M6PR				NCF2		THBS3	

Table 4
Members of innate immune receptor pathways whose phosphorylation were significantly (p-value ≤ 0.05) affected by *Salmonella* infection at 1.5, 3, and 7 hpi.

S. Enteritidis						S. Heidelberg					
1.5 hpi		3 hpi		7 hpi		1.5 hpi		3 hpi		7 hpi	
NOD-like receptor signaling pathway											
CARD9	NLRP3	BIRC3	MAPK1	BIRC3	MAPK1	CHUK	NLRP3	BIRC3	MAPK14	BIRC3	NFKB1
CASP1	TAB3	CARD9	NFKB1	CARD9	NFKB1	HSP90B1	TAB1	CASP8	MAPK8	CARD9	NFKBIA
CHUK		CASP1	NFKBIA	CASP1	NLRP3	MAP3K7	TAB3	CHUK	NFKB1	CASP1	NLRP3
HSP90B1		CASP8	TAB3	CASP8	PSTPIP1	MAPK1		HSP90AB1	NLRP3	CHUK	TAB3
MAPK8		CHUK	TRAF6	CHUK	TAB1	NFKB1		MAP3K7	PSTPIP1HSP90B1		
NFKB1		HSP90AB1		MAP3K7	NFKBIA		MAPK1	TRAF6	MAPK1		
Toll-like receptor signaling pathway											
AKT1	TLR3	AKT3	TIRAP	AKT1	PIK3CB	AKT1	TAB1	AKT1	PIK3CB	AKT1	PIK3R1
AKT3		CASP8	TLR1	AKT3	PIK3CD	CHUK	TIRAP	CASP8	PIK3CD	AKT3	STAT1
CHUK		CHUK	TLR3	CASP8	PIK3CG	IFNAR1	TLR3	CHUK	PIK3CG	CHUK	TBK1
IKBKE		IL12B	TLR5	CHUK	PIK3R1	JUN	TLR5	FOS	PIK3R1	IFNAR1	TIRAP
JUN		JUN	TOLLIP	FOS	PIK3R2	MAP2K1		IL12B	PIK3R2	IKBKE	TLR1
MAP2K3		MAP2K1	TRAF6	IFNAR1	STAT1	MAP2K2		JUN	TBK1	JUN	TLR3
MAP2K4		MAP2K3		IKBKE	TAB1	MAP2K4		MAP2K1	TIRAP	MAP2K1	TLR6
MAP3K8		MAP3K8		IL12B	TBK1	MAP3K7		MAP2K2	TLR1	MAP2K2	TLR7/8
MAPK8		MAPK1		JUN	TIRAP	MAP3K8		MAP2K3	TLR3	MAP2K4	
NFKB1		NFKB1		MAP2K1	TLR1	MAPK1		MAP2K4	TLR5	MAP3K8	
PIK3CB		NFKBIA		MAP2K2	TLR3	NFKB1		MAP3K7	TLR7/8	MAPK1	
PIK3CG		PIK3CB		MAP2K4	TLR6	NFKBIA		MAP3K8	TOLLIP	NFKB1	
PIK3R1		PIK3CD		MAP3K7	TLR7/8	PIK3CB		MAPK1	TRAF6	NFKBIA	
PIK3R2		PIK3CG		MAP3K8	TOLLIP	PIK3CD		MAPK14		PIK3CB	
TIRAP		PIK3R1		MAPK1		PIK3CG		MAPK8		PIK3CD	
TLR1		TBK1		NFKB1		PIK3R2		NFKB1		PIK3CG	
RIG-I-like receptor signaling pathway											
MAPK8		TBK1	CASP8	TBK1	CASP8	NFKBIA		TBK1	NFKB1	TBK1	MAP3K1
IKBKE		IL12B	CHUK	IL12B	CHUK	NFKB1		MAPK8	TRAF6	NFKBIA	
NFKB1		NFKBIA		IKBKE	MAP3K7	CHUK		IL12B	CASP8	IKBKE	
CHUK		NFKB1		TRAF2	MAP3K1	MAP3K7		TRAF2	CHUK	NFKB1	
		TRAF6		NFKB1				MAPK14	MAP3K7	CHUK	
Cytosolic DNA-sensing pathway											
CASP1	NFKB1	CASP1	NFKBIA	CASP1	NFKB1	CHUK		CHUK		CASP1	NFKB1
CHUK		CHUK	TBK1	CHUK	TBK1	NFKB1		NFKB1		CHUK	NFKBIA
IKBKE		NFKB1		IKBKE		TBK1		TBK1		IKBKE	TBK1

consensus sequence corresponding to human TLR4 phosphorylation site. However, STRING database generated GO Biological Process indicate that TLR4 pathway is very much involved in the *Salmonella* infection; as there were 22 (p = 3.7E-17), 26 (p = 1.2E-21), and 33 (p = 1.3E-29) peptides and 23 (p = 4.6E-19), 27 (p = 1.5E-22), and 26 (p = 5.0E-22) peptides associated with TLR4 pathway affected by cells infected with *S*. Enteritidis and *S*. Heidelberg at 1.5, 3, and 7 hpi, respectively. Additionally, STRING generated GO Biological Process also indicate participation of both MyD88 dependent and independent pathways in the HD11 cell response to *Salmonella* infection (not shown). Interestingly, some TLRs, such as viral RNA receptors TLR3 and 7, that may not be directly involved in macrophage cells' response to *Salmonella* infection were also found to be significantly affected. The results imply that once chicken macrophages encounter pathogens, they mobilize not one, but an array of innate immune defense mechanism. From these peptides listed in Table 4, three major kinase groups, including AKT kinases, phosphoinositide-3-kinases (PI3Ks), and MAP kinases are clearly shown to be critical in convey signals from TLRs to the nuclear transcription factors NFκB and AP-1 (JUN and FOS).

4.4. *Salmonella* infection down-regulates CARD9 phosphorylation in NOD-like receptor pathway

As cytosolic sensors of intracellular PAMPs, the nucleotide oligomerization domain (Nod)-like receptors (NLRs) is another innate immune receptor family that plays an important role in immune defense against intracellular bacterial infection. There are 22 NLRs reported in human; while chickens have NOD1, but not NOD2, and have only 5 NLRs in total (Laing et al., 2008; Lian et al., 2012; Tao et al., 2015; Ye et al., 2015). Among them, NOD1 and NOD2 have been well documented to recognize the structural component of bacterial peptidoglycan (Motta et al., 2015). Recent studies indicate that NOD1 and NOD2 are also involved in regulation of inflammation and clearance of *S*. Typhimurium in mouse mucosal dendritic cells and tissue; NOD1 deficiency impairs clearance of the bacteria (Le Bourhis et al., 2009; Geddes et al., 2010). In the present study, the peptide array data imply the involvement of NLR pathway in chicken macrophages' response to *Salmonella* infection (Table 4). Caspase recruitment domain-containing protein (CARD) 9 is the adaptor protein that interacts with NOD1 and kinase RIP2 to regulate the cell apoptosis and to signal activation of transcription factor NFκB and MAP kinase p38 and JNK (Bertin et al., 2000; Ruland, 2008). Phosphorylation of T231 of murine CARD9 by Syk kinase is required for CARD–CARD domain interaction to form Card9-Bcl10 complex (Strasser et al., 2012), indicating T231 phosphorylation is likely important for interaction with other CARD-containing proteins, such NOD1, NOD2, and RIP2 kinase. However, the peptide array data show that CARD9 (T238) phosphorylation in HD11 cells is mostly down-regulated during the infection; the phosphorylation changes (fold) at 1.5, 3, and 7 hpi are −1.14 (p = 0.00), 1.03 (p = 0.04), and −1.19 (p = 0.00) for *S*. Enteritidis and −1.02 (p = 0.17), −1.01 (p = 0.38), and −1.14 (p = 0.00) for *S*. Heidelberg. We speculate that *Salmonella* may interfere with NOD-receptor mediated cellular response by inhibiting the CARD9 function. Particularly, significantly down-regulating CARD9 phosphorylation was shown at all three time points post infection from cells infected with *S*. Enteritidis.

Table 5
Members of immune response signaling pathways whose phosphorylation were significantly (p-value ≤ 0.05) affected by *Salmonella* infection at 1.5, 3, and 7 hpi.

S. Enteritidis					S. Heidelberg						
1.5 hpi		3 hpi		7 hpi		1.5 hpi		3 hpi		**7 hpi**	
MAPK signaling pathway											
AKT1	NTRK1	AK3	NFKB2	AKT1	MAP3K14	AKT1	NFATC2	AKT1	MAPK1	AKT1	PAK1
AKT3	PAK2	ARRB1	NTRK1	AKT3	MAP3K3	ARRB1	NFKB1	ARRB2	MAPK14	AKT3	PAK2
ARRB1	PDGFRA	ARRB2	PAK1	ARRB1	MAP3K7	ARRB2	NTRK1	ATF2	MAPK8	ARRB1	PDGFRA
ARRB2	PDGFRB	ATF2	PAK2	ATF2	MAP3K8	CASP3	PAK1	BRAF	MKNK1	ATF2	PDGFRB
CASP3	PLA2G4A	BRAF	PDGFRA	BRAF	MAPK1	CHUK	PAK2	CASP3	NFATC2	BRAF	PRKACA
CHUK	PRKACA	CHUK	PDGFRB	CASP3	MAPK2	CRK	PDGFRA	CHUK	NFKB1	CASP3	PRKCA
CREB	PRKCA	CREB	PRKACA	CHUK	MAPK3	DUSP1	PRKACA	CREB	NTRK1	CHUK	RPS6KA1
CRKL	RAF1	CRK	PRKCA	CREB	MAPK5	DUSP10	PRKCA	CRK	PAK1	CRK	RPS6KA3
EGFR	RASGRP3	CRKL	RAF1	CRK	MKNK1	DUSP6	RAF1	DUSP1	PAK2	DUSP1	RPS6KA5
FGF20	RPS6KA1	DUSP1	RASGRP3	CRKL	NFATC2	EGFR	RASGRP3	EGFR	PDGFRA	EGFR	SOS1
FGFR2	RPS6KA3	DUSP10	RPS6KA1	DUSP1	NFKB1	FGF20	RPS6KA1	FGFR2	PDGFRB	FGFR1	STMN1
FGFR3	RPS6KA5	EGFR	RPS6KA3	DUSP10	NFKB2	FGFR1	RPS6KA3	FGFR3	PLA2G4A	FGFR2	TAOK1
FGFR4	SOS1	FGF20	RPS6KA5	EGFR	NTRK1	FGFR2	RPS6KA5	FGFR4	PRKACA	FGFR4	
HSPA8	STMN1	FGFR2	SOS1	FGF20	PAK1	FGFR3	STMN1	FOS	PRKCA	HSPA8	
JUN	TAOK1	FGFR3	TRAF6	FGFR1	PAK2	HSPA8	TAB1	HRAS	RPS6KA1	JUN	
MAP2K3		FGFR4		FGFR2	PDGFRA	JUN		HSPA8	RPS6KA3	MAP2K1	
MAP2K4		GRB2		FGFR3	PDGFRB	MAP2K1		HSPB1	RPS6KA5	MAP2K2	
MAP2K5		HSPA8		FGFR4	PRKACA	MAP2K2		JUN	SOS1	MAP2K4	
MAP3K11		JUN		FOS	PRKCA	MAP2K4		MAP2K1	STMN1	MAP2K5	
MAP3K14		MAP2K1		GRB2	RAF1	MAP2K5		MAP2K2	TAOK1	MAP3K1	
MAP3K3		MAP2K3		HSPA8	RASGRP3	MAP3K11		MAP2K3	TRAF2	MAP3K14	
MAP3K8		MAP2K5		HSPB1	RPS6KA1	MAP3K3		MAP2K4	TRAF6	MAP3K8	
MAPK8		MAP3K11		JUN	RPS6KA3	MAP3K5		MAP2K5		MAPK1	
MAPK2		MAP3K3		MAP2K1	RPS6KA5	MAP3K7		MAP3K11		MAPK2	
MAPK3		MAP3K8		MAP2K2	SOS1	MAP3K8		MAP3K14		MAPK5	
MKNK1		MAPK1		MAP2K4	STMN1	MAPK1		MAP3K3		MKNK1	
NFATC2		MAPK2		MAP2K5	TAB1	MAPK2		MAP3K5		NFKB1	
NFKB1		MAPK3		MAP3K1	TAOK1	MAPK3		MAP3K7		NFKB2	
NFKB2		NFKB1		MAP3K11	TRAF2	MKNK1		MAP3K8		NTRK1	
Jak-STAT signaling pathway											
AKT1	PIK3R2	AKT3	SOS1	AKT1	PIK3CB	AKT1		AKT1	PIM1	AKT1	SOS1
PIK3CB	STAT3	CBL	STAM	AKT3	PIK3CD	CCND1		CBL	SOCS3	AKT3	STAT1
PIK3R1		EP300	STAM2	CBL	PIK3CG	IFNAR1		CCND1	SOS1	IFNAR1	STAT4
JAK1		GRB2	STAT4	CCND1	PIK3R1	IL23R		EP300	STAM	IL23R	STAT5B
STAT5B		IL12B	STAT5B	EP300	PIK3R2	IL6ST		IL12B	STAM2	IL6ST	
AKT3		IL23R		GRB2	PIM1	JAK2		IL6ST	STAT3	JAK1	
JAK2		IL6ST		IFNAR1	SOCS3	PIK3CB		JAK1	STAT4	JAK2	
SOS1		JAK1		IL12B	SOS1	PIK3CD		JAK2	STAT5B	PIK3CB	
PIK3CG		JAK2		IL23R	STAT1	PIK3CG		PIK3CB		PIK3CD	
IL6ST		PIK3CB		IL6ST	STAT3	PIK3R2		PIK3CD		PIK3CG	
PIM1		PIK3CD		IL7R	STAT4	STAT5B		PIK3CG		PIK3R1	
SOCS3		PIK3CG		JAK1	STAT5B			PIK3R1		PIM1	
CCND1		PIK3R1		JAK2				PIK3R2		SOCS3	

4.5. *Salmonella* infection reduces tyrosine phosphorylation of NLRP3

The NLRP3 is the major component of inflammasome, a multi-protein oligomer consisting of NLRP3, adaptor protein ACS, and caspase-1. The inflammasome is part of the innate immune system and is activated in response to microbial infection and cellular stress signals in myeloid cells. Activated caspase-1 in NLRP3 inflammasome converts pro IL-1β and pro IL-18 into their active forms which can then be released from the cell to mediate inflammatory response (Baroja-Mazo et al., 2014). At normal physiological state, NLRP3 activity is negatively controlled by phosphorylation at tyrosine residues and activation is accomplished by reducing phosphorylation. In human peripheral blood mononuclear cells, 60%–80% of NLRP3 was tyrosine phosphorylated in nonactivated cells, and the level dropped to below 10% upon activation (Spalinger et al., 2016). The tight control of the tyrosine phosphorylation of NLRP3 is critical for preventing excessive inflammatory responses. In the present study, significant reduction of tyrosine phosphorylation at T24 (corresponding to human NLRP3 T233) was first observed at 1.5 hpi for *S*. Enteritidis (-1.07 fold, $p = 0.02$) and 3 hpi for *S*. Heidelberg (-1.09 fold, $p = 0.00$); both *Salmonella* strains induced further reduction of NLRP3 tyrosine phosphorylation at 7 hpi, with -1.40 ($p = 0.00$) for Enteritidis and -1.22 ($p = 0.00$) for Heidelberg. These results indicate NLRP3 likely plays a role in *Salmonella* infection induced inflammatory response in chicken macrophages.

4.6. MAP Kinases/PI3 K/AKT plays a central role in chicken macrophage response to Salmonella infection

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases. The MAPK family is consisted of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK); each of these MAPKs are activated sequentially by specific MAPK kinases (MAP2Ks) and MAPK kinase kinases (MAP3Ks) (Zhang and Dong, 2005). They play a critical role in the innate immune response to pathogens by activating nuclear transcription factors to increase expression of genes required for pro-inflammatory responses, such as cytokines and chemokines. In the present study, the MAPK signaling pathway emerged as one of the most significantly affected by *Salmonella* infection among KEGG pathways and GO-Biological processes generated by the STRING database. As shown in Table 5, a large number of peptides representing members of the MAPK pathway show significantly altered phosphorylation at all three time points during the infection. These proteins belong to various signaling groups in the cascade, including up-stream receptors (FGFR, PDGFR, and ARRB) and

kinases (AKTs, PAKs, PKA, PKC, RAF1, TAB); MAPK family {MAP3Ks, MAP2Ks, and MAPKs [ERK(MAPK1, 2, 3), JNK (MAPK8), and p38 (MAPK14)}; and down-stream target kinases [cPLA2, MKNK, RPS6 K, MAPKAPK2 (MAPK2), MSK (RPS6KA5)]. Many transcription factors that are regulated by MAPK family also display significantly altered phosphorylation, including AP-1 (JUN and FOS), ATF2, NFAT1/2, NFκB1/2, and CREB. The effect of *Salmonella* infection on host cell MAPK pathway was dynamic and extensive as revealed by the peptide array data. The result underscores the importance of MAPKs in chicken macrophage response to *Salmonella* infection.

The array data also indicate that phosphatidylinositol-3 kinases (PI3Ks) and protein kinase B (PKB), also known as AKT, are critical players in chicken macrophage response to *Salmonella* infection; as phosphorylation of many PI3Ks and AKT1/3 were significantly altered during infection (Tables 4 and 5). These results clearly indicate that MAPKs, PI3Ks, and AKTs are at the center of cross-talk linking various pathways of the kinase network which convey signals from receptors to wide range of cellular functions. Similarly, extensive changes in many of the same kinases involved in the T-cell receptor, mTOR/AKT, and JAK-STAT signaling pathways have reported in gut tissues of *Salmonella* infected chickens (Kogut et al., 2016). It is reasonable to speculate that macrophage cells infected with *Salmonella* at the intestinal tissue may have contributed the reported outcome results.

5. Conclusion

The chicken specific peptide array-based kinome analysis used in the present study has proven to be a powerful tool to undercover a complex interaction between *Salmonella* and chicken macrophage cells. The data provided a global view of dynamic phosphorylation changes in proteins involved in the cellular kinase network during the *Salmonella* infection of chicken macrophage cells, which identify critical cellular processes and signaling pathways that determine the outcome of the infection. Significant difference in kinome response identified between the two strains *S.* Enteritidis and *S*. Heidelberg provided evidence that explains different *Salmonella* strains interact differently with the host. Many pathways involved in immunity, signal transduction, cellular process, and metabolism were significantly altered, in some case differentially, during the infection by the two *Salmonella* strains. Particularly, the lysosome process which was differentially affected by the two *Salmonella* strains during the infection may be a key factor responsible for the reported differences in intracellular survivability and macrophage NO response. MAPKs, PI3K3, and AKTs appear to play a center role in coordinating various pathways in host response to *Salmonella* infection. Protein kinases have been shown to associate with a large number of diseases and are increasingly targeted as therapeutic intervention points. *Salmonella* are known to manipulate the host kinase network to gain entry and survival inside the host cells. Our data provide information that may facilitate discovery of novel molecules that target host cell kinase as alternatives to antibiotics to control *Salmonella* carriage in poultry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2017.11.002.

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